

AD\_\_\_\_\_

Award Number: W81XWH-08-1-0446

TITLE: Role of BRCA2 in the Expressions of IRF9-Regulated Genes in Human Breast Cells

PRINCIPAL INVESTIGATOR: Gautam Chaudhuri, Ph.D.

CONTRACTING ORGANIZATION: Meharry Medical College  
~~Meharry Medical College~~

REPORT DATE: July 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved  
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE July 2011			2. REPORT TYPE Final		3. DATES COVERED 1 July 2008 – 31 July 2011	
4. TITLE AND SUBTITLE  Role of BRCA2 in the Expressions of IRF9-Regulated Genes in Human Breast Cells			5a. CONTRACT NUMBER			
			5b. GRANT NUMBER W81XWH-08-1-0446			
			5c. PROGRAM ELEMENT NUMBER			
6. AUTHOR(S)  Gautam Chaudhuri  E-Mail: gchaudhuri@mmc.edu			5d. PROJECT NUMBER			
			5e. TASK NUMBER			
			5f. WORK UNIT NUMBER			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  Meharry Medical College Nashville, TN 37208			8. PERFORMING ORGANIZATION REPORT NUMBER			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)			
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)			
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT  We hypothesize that BRCA2 facilitates the formation and/or the function of the ternary ISGF3 complex and thus, functional BRCA2 protein is essential for the antiproliferative effects of type I interferons against human breast tumor cells. <b>Specific aims:</b> <b>(A)</b> To evaluate further the structural and functional interactions of BRCA2 with the members of the ISGF3 complex (STAT1, STAT2 and IRF9) in the human breast cells. <b>(B)</b> To evaluate the antiproliferative effects of BRCA2 over expression in the human breast cells with or without knock down of the IRF9 protein by RNA interference. <b>(C)</b> To evaluate the antiproliferative effects of type I interferons against tumors developed by BRCA2 positive and BRCA2 negative human breast tumor cells in the nude mice xenograft model.						
15. SUBJECT TERMS Breast cancer, BRCA2, Interferon alpha, Interferon beta, STAT1, STAT2, IRF9, ISGF3						
16. SECURITY CLASSIFICATION OF:  a. REPORT U			17. LIMITATION OF ABSTRACT  UU	18. NUMBER OF PAGES  18	19a. NAME OF RESPONSIBLE PERSON USAMRMC	
b. ABSTRACT U			19b. TELEPHONE NUMBER (include area code)			
c. THIS PAGE U						

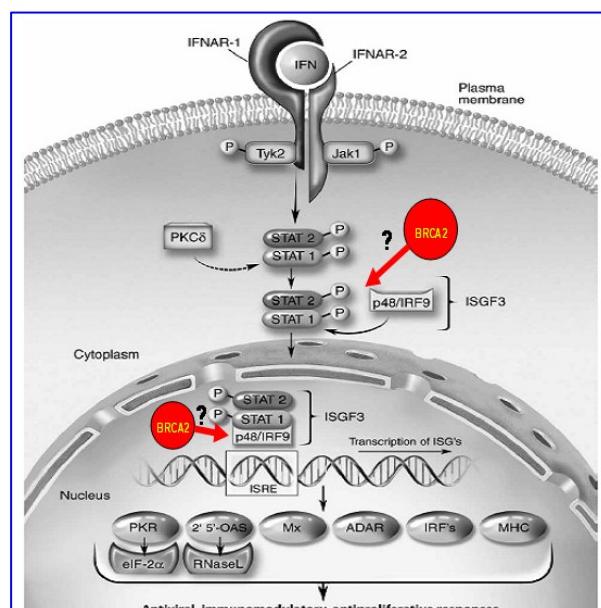
## **Table of Contents**

	<u>Page</u>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>5</b>
<b>Key Research Accomplishments.....</b>	<b>14</b>
<b>Reportable Outcomes.....</b>	<b>14</b>
<b>Conclusion.....</b>	<b>16</b>
<b>References.....</b>	<b>16</b>
<b>Appendices.....</b>	<b>18</b>

**Role of BRCA2 in the expressions of IRF9-regulated genes in human breast cells PI:  
Gautam Chaudhuri, PhD**

## INTRODUCTION

BRCA2, a tumor suppressor whose inactivation is associated with hereditary breast and ovarian cancer predisposition, is essential for DNA repair in mammalian cells [1-5]. BRCA2-deficient cells are defective in the repair of DNA double-strand breaks by error-free homologous recombination [5, 6], allowing error-prone repair processes to create gross chromosomal rearrangements that may promote carcinogenesis [6]. The role of BRCA2 in homologous recombination has been linked to its functions in the regulation of RAD51, a RecA-related recombinase that forms the nucleoprotein filaments on damaged DNA that are crucial to recombinational repair [7, 8]. BRCA2 binds directly to RAD51 through 6 of the 8 BRC repeats, ~30 amino acid motifs encoded within the central exon 11 region of all known mammalian BRCA2 genes [6, 9-12]. In addition to its role in DNA double-strand break repair, BRCA2 also plays a role in stabilization of stalled DNA replication forks, cytokinesis, transcription regulation, mammalian gametogenesis, centrosome duplication, and suppression of cell proliferation [6]. However, how BRCA2 mutations predispose women specifically to breast and ovarian cancer remains undefined.



**Fig. 5. Type I interferon (IFN) pathway. The predicted roles of BRCA2 (in red) are indicated.**

[13].

We found that transient ablation of the breast cancer susceptibility gene BRCA2 in the human breast cells (Fig. 1) impairs the expressions of many type I interferon regulated genes. Thus, it appears that type I interferons need functional BRCA2 for their actions [14]. Thus, negative regulation of IFN-induced genes in BRCA2-ablated breast cells may reflect another growth regulatory role of BRCA2.

The interferons (IFNs), in addition to their well-known antiviral activities, have important roles in the control of cell proliferation and are effective agents for the treatment of malignant diseases. IFNs not only regulate cell growth and division but also influence cell survival through their effects on apoptosis.  $\alpha$  and  $\beta$  interferons are type I IFN proteins with antitumor activity [15, 16]. They down regulate oncogene expression and induce tumor suppressor genes, which result in antiproliferative activity. The classic pathway induced by type I IFNs involves the interaction

of the IFN with two-receptor subunits, IFNAR-1 and -2, which are associated with TYK-2 and JAK-1, respectively [17-19]. TYK-2 and JAK-1 phosphorylate tyrosine residues on the receptor that provide docking sites for the src-homology-2 (SH2) domains of STATs in a cell type specific manner [15, 16]. Once phosphorylated, STATs are released from the receptor and form heterodimers. In response to Type I IFNs, STAT2 is recruited to the IFNAR1 chain, where it is phosphorylated by TYK-2 and serves as a lure for STAT1 [15, 16]. Once released from the receptor, the resulting STAT1:STAT2 heterodimer associates with IRF9, a DNA binding protein (also called p48), forming a complex named IFN-stimulated gene factor-3 (ISGF3). After formation, ISGF3 translocates to the nucleus where it binds to the IFN-stimulated response elements (ISRE) upstream of IFN response genes and initiates transcription (Fig. 1).

We hypothesize that BRCA2 facilitates the formation and/or the function of the ternary ISGF3 complex and thus, functional BRCA2 protein is essential for the antiproliferative effects of type I interferons against human breast tumor cells.

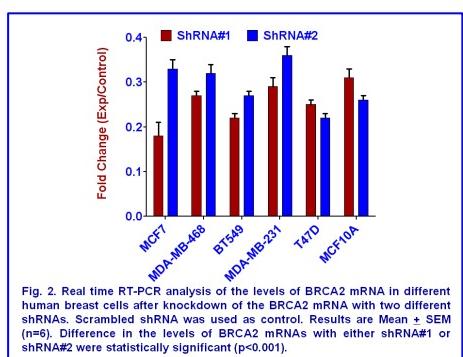
**Specific aims to verify the hypothesis are:** (A) To evaluate further the structural and functional interactions of BRCA2 with the members of the ISGF3 complex (STAT1, STAT2 and IRF9) in the human breast cells. (B) To evaluate the antiproliferative effects of BRCA2 over expression in the human breast cells with or without knock down of the IRF9 protein by RNA interference. (C) To evaluate the antiproliferative effects of type I interferons against tumors developed by BRCA2 positive and BRCA2 negative human breast tumor cells in the nude mice xenograft model.

## BODY

### Task outlined in the approved Statement of Work for this period of the project

#### Task#1

**To evaluate further the structural and functional interactions of BRCA2 with the members of the ISGF3 complex (STAT1, STAT2 and IRF9) in the human breast cells. (Months 1-18)**



**1. We knocked down BRCA2 gene expression in the following human breast cancer cells: MCF7, MDA-MB-468, BT549, MDA-MB-231, T47D and MCF10A.** We used Sigma Mission validated lentiviral shRNA particles to knockdown the BRCA2 mRNAs. The puromycin-resistant clones are evaluated for BRCA2 mRNA and protein by qRT-PCR (Applied Biosystems) and Western blotting analysis (R & D Systems), respectively. The BRCA2 mRNA levels decreased 70-85% (Fig. 2) and the protein

levels decreased 55-65% (Fig. 3).

**2. We evaluated the subcellular localization of ISGF3 complex in the control and BRCA2/KD cells.** Our preliminary data suggested that BRCA2 binds with the components of ISGF3. The first question we asked is whether the nuclear localization of ISGF3 is affected in BRCA2 deficiency. We determined the subcellular locations of ISGF3 components by immunofluorescence confocal microscopy. We used commercially

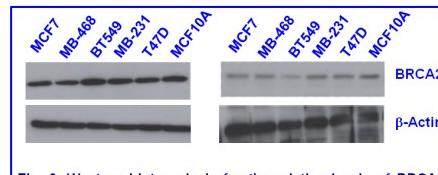
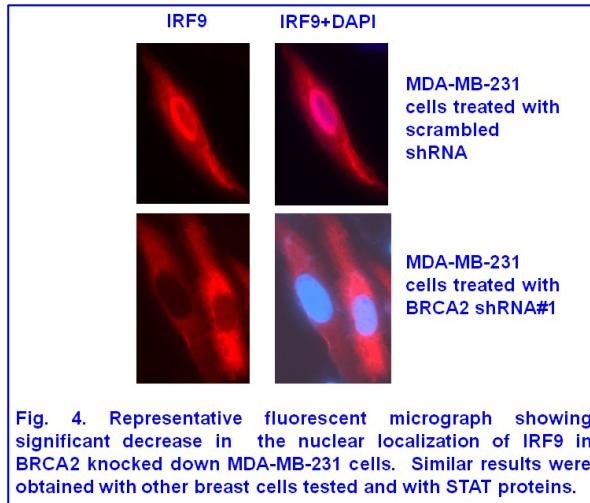


Fig. 3. Western blot analysis for the relative levels of BRCA2 protein in different human breast cells lines before and after shRNA-mediated knockdown of this mRNA in these cells.  $\beta$ -Actin was used as loading controls.

available (Santa Cruz Biotech) antibodies against the ISGF3 component proteins for these experiments. For the detection of IRF9 we used ISGF-3 $\gamma$  p48 (B-18): sc-101070, a mouse monoclonal antibody. For Stat1 we used Stat1 p84/p91 (H-95) sc-98783 rabbit polyclonal



**Fig. 4.** Representative fluorescent micrograph showing significant decrease in the nuclear localization of IRF9 in BRCA2 knocked down MDA-MB-231 cells. Similar results were obtained with other breast cells tested and with STAT proteins.

of human origin for the immunofluorescence detection of the BRCA2 protein. We are evaluating whether phosphorylation of the STAT proteins (Tyr-701 in Stat-1 $\beta$  p84 and Stat1 $\alpha$  p91 and Tyr-690 in Stat-2) are also required for their binding with BRCA2 by co-immunoprecipitation and Western blotting with phospho-specific antibodies. We are expressing N-terminal GST-tagged Stat-2 protein or Stat-2 protein with mutation (Tyr-Ala) at the Tyr-690 in breast cells like MCF7 and T47D. The Stat-2 protein without Tyr-690 will not be able to be phosphorylated. We will evaluate whether this protein can form complex with BRCA2.

**3. We have developed a BRCA2 derivative that lacks only the nuclear localization signals but not the other C-terminal domains.** We started with the pCINBRCA2WT plasmid, a clone of full-length human BRCA2 protein in pcDNA3 vector, a gift from Prof. Mien-Chie Hung of

MD Anderson. We designed primers to amplify the whole plasmid except the two nuclear localization signals at the C-terminus of the BRCA2 cDNA following long-distance PCR protocols. We introduced stop codon and Fse I (NEB) site in the primers. Fse I site is absent in the vector and the insert DNA. We digested the purified amplified DNA with Fse I and relegate the plasmid DNA. This plasmid expressed a C-terminal 153 amino acid residue truncated BRCA2 protein lacking the two nuclear localization signals (Fig. 5). We expressed this NLS-deleted version of BRCA2 in Capan-1 cells and currently characterizing

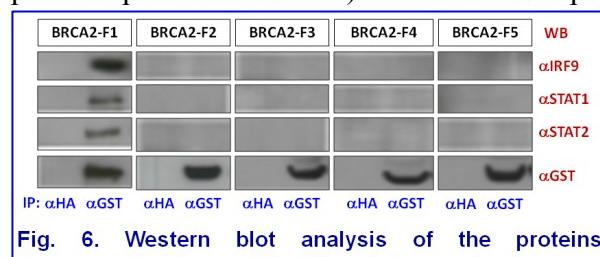
the recombinant clones. We will employ these recombinant cells to further verify the need for nuclear localization of the BRCA2 protein for the activation of the ISG15 gene promoter. We

will also use these cells for subcellular fractionation and co-immunoprecipitation to evaluate by Western blotting whether BRCA2-ISGF3 complex is formed in the cytosol. These data will be generated soon.

### Task#2

**To evaluate the antiproliferative effects of BRCA2 over expression in the human breast cells with or without knock down of the IRF9 protein by RNA interference. (Months 16-30)**

**1. We evaluated the ability of different domains of human BRCA2 protein in pulling down the components of the BRCA2/ISGF3 complex.** Human BRCA2 is a large protein (3418 amino acids). We have amplified 5 consecutive fragments from the human BRCA2 ORF (in plasmid pCINBRCA2WT). There are five peptide fragments: the first 4 fragments are from the



**Fig. 6. Western blot analysis of the proteins immunopulled down with different fragments of human BRCA2 protein in MDA-MB-231 cells. HA antibody was used as a control in the pulled down experiment. Similar results were obtained with BT549 cells.**

N-terminal end and have 683 amino acids (ORF ~2055 bp with start and stop codons) and the 5<sup>th</sup> C-terminal fragment has 686 amino acids. We have cloned these PCR amplified products into N-terminal GST-tagged protein expression vector [pFN2K (GST) Flexi® Vector]. We expressed these N-terminal GST-tagged BRCA2 peptide fragments in human breast cells (Fig. 6). By GST-pull down assays we found that only the N-terminal 683 amino acid fragment of

BRCA2 was able to pull down all three components of the ISGF3 complex (Fig. 6). We conclude that BRCA2 interacts with the ISGF3 complex perhaps through the N-terminal domains. As it does not interact with its BRC repeats with ISGF3, ISGF3 will not compete with RAD51.

#### **BRCA2-F1**

MPIGSKERPTFFEIFKTRCNKADLGPISLNWFEELSSEAPPYNSEPAEESEHKNNNYEPNLFKT  
PQRKPSYNQLASTPIIFKEQGLTLPLYQSPVKELDKFKLDLGRNVPNRSRKSLRTVKTMDQAD  
DVSCPILLNSCLSES PVVLQCTHVT PQRDKSVVCGSLFHTPKFVKGRQTPKHISESLGAEVDPDM  
SWSSSLATPPTLSSTV LIVRNEEASET VFPHD TTANVKS YFSNHDESLKKNDRFIASVTDSENT  
NQREAASHGFGKTS GNSFKVNSCKDHIGKSMPNVLEDEVYETVVDTSEEDSFSLCF SKCRTK NL  
QKVRTSKTRKKI FHEANADECEKSKNQVKEKYSFVSEVEPNDT DPLDSNVANQKPFESGSDKIS  
KEVVPSLACEWSQLTLSGLNGAQMEKIPLLHISSCDQNISEKDLLDTE NKRKKDFLTSEN SLPR  
ISSLPKSEKPLNEETV VVNKRDEEQHLESHTDCILAVKQAISGTSPVASSFQGI KKSIFRI RESP  
KETFNASFSGHMTDPNFKKETEASESGLEIHTVCSQKEDSLCPNLIDNGSWPATTQNSVALKN  
AGLISTLKKTNKFIYAIHDETSYKGKKIPKDQKSELINCSAQFEANAFEAPLT FANADSGLLH  
SSVKRSCSQNDSEEPTLSLTSSFGTILRKCSRNETCSNNTVIS

#### **BRCA2-F2**

QLDYKEAKCNKEKLQLFITPEADSLSCLQEGQCENDPKSKVSDIKEEVLAACHPVQHSKVE  
YSDTDFQSQKSLLYDHENASTLILPTPSKDVLSNLVMISRGKESYKMSDKLKGNNNYESDVELTK  
NIPMEKNQDVCALNENYKNVELLPPEKYM RVASPSRKVQFNQNTNLRVIQKNQEETTSISKITV  
NPDSEELFSDNENN FVQVANERNNLALGNTKELHETDLTCVNEPIFKNSTMVL YGDTGDKQAT  
QVSIIKKDLVYVLAEEENKNSVKQHIKMTLGQDLKSDISLNIDK IPEKNNNDYMNKWAGLLGPISNH  
SFGGSFRTASNKEIKLSEHNICKSKMFFKDIEE QYPTSLACVEIVNTLALDNQKKLSKPQSINT

VSAHLQSSVVSDCKNSHITPQMLFSKQDFNSNHNLTPSQAEITELSTILEESGSQFEFTQFR  
KPSYILQKSTFEVPENQMTILKTTSEECRDADLHVIMNAPSIGQVDSSKQFEGTVEIKRKFAGL  
LKNDCNKSASGYLTDENEVGFRGFYSAHTKLNVSTEALQAVKLFSDIENISEETS AEVHPIS  
LSSSKCHDSVVSFMKIENHNDKTVEKNNKCQLILQNNIEMTTGTFVEEITENYKRNTENEDNK  
YTAASRN SHNLEFDGSDSSKNDTVCIH KDET DLLFTDQHNICL

#### **BRCA2-F3**

KLSGQFMKEGNTQIKE DLSDLTFLEVAKAQEA CHGNTSNKEQLTATKTEQNIKDFETSDTFFQT  
ASGNISVAKESFNKIVNFFDQKPEELHNFSLNSELHSDIRKNKMDILSYEETDIVKHKILKES  
VPVGTGNQLVTFOGQPERDEKIKEPTLLGFHTASGKKVKIAKESLDKVKNLFDEKEQGTSEITS  
FSHQWAKTLKYREACKDLELACETIEITAAPKCKEMQNSLNNDKNLVSIE TVVPPKLLSDNLCR  
QTENLKTTSKSIFLKVKVHENVEKETAKSPATCYTNQSPYSVIENSALAFYTCSRKT SVSQTSL  
LEAKKWLREGIFDGQPERINTADYVGNYLYENNNSNSTIAENDKNHLSEKQDTYLSN SSMNSNSYS  
YHSDEVYNDSGYLSKNKLD SGIEPVLKVNVEDQKNTSF SKVISNVKDANAPQTVNEDICVEELV  
TSSSPCKNKNAAIKLSISNSNNFEVGPFAFRIASGKIVCVSHETIKVKDIFTDSFSKVIKENN  
ENKSKICQTKIMAGCYEALDDSEDILHNSLDNDECSTHSHKFADIQSEEILQHNQNMSGLEKV  
SKISP C DVS LETSDICKCSIGKLHKSVSSANTCGIFSTASGKSVQVSDASLQNARQVFSEIEDS  
TKQVFSKVLFKSNEHSDQLTREENTAIRTP EHLISQKGFSY NV

#### **BRCA2-F4**

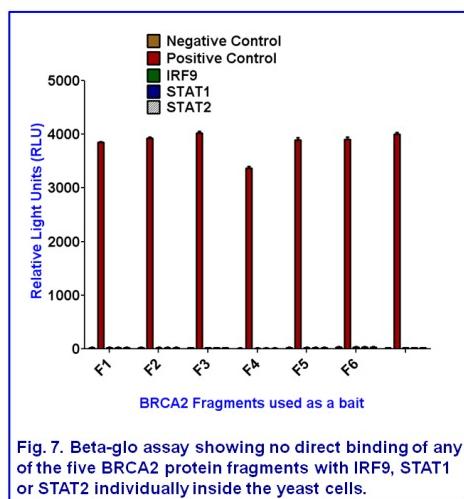
VNSSAFSGFSTASGKQVSILESSLHKVGVLEEFDLIRTEHSLHYSPTSRQNVSKILPRVDKRN  
PEHCVNSEMEKTCSEFKLSNNLNVEGGSSENNHSIKVSPYLSQFQQDKQQLVLGTVKSLVENI  
HVLGKEQASPKNVKMEIGKTETFSDVPVKTNIEVCSTY SKDSEN YFETEAVEIAKAFMEDDELT  
DSKLPSHATHSLFTCPENEEMVLSNSRIGKRRGEPLILVGEPSIKRNLLNEFDRII ENQEKS LK  
ASKSTPDGTIKDRRLFMHHVSLEPITCVPFR TTKERQEIQNP NFTAPGQEF LSKSHLYEHTL  
KSSSNLA VSGHPFYQVSATRNEKMRHLITTGRPTKVFVPPFKTKSHFHRVEQCVRNINLEENRQ  
KQNI DGHGSDDS KNKINDNEIHQFNKNNSNQAAAVFTKCEEEPLDLITS LQNARDI QDMRIKK  
KQRQRF PQPGS LYLA KTSTLPRISLKA AVGGQVPSACSHKQLYTYGVSKHC IKINSKNAESFQ  
FHTEDYFGKESLWTGKG IQLADGGW LIPS NDGKAGKEFYRALCDTPGVDPKLISRIWVY NHYR  
WIIWKLAAMECAF PKEFANRCLS PERVLLQLKYRYDTEIDRSRRSAIKKIMERDDTA AKTLVLC  
VSDIISLSANISETSSNKTSSADTQV AIIELTDG WYAVKAQL

#### **BRCA2-F5**

DPPLLAVLKNGRLTVGQKII LHGAELVGSPDACTPLEAPESLMLKISANSTRPARWYTKLGFFP  
DPRPFPLPLSSLFSDGGNVGCVDVI IQRAYPIQWMEKTSSGLYIFRNERE EEEKA AKYVEAQQK  
RLEALFTKIQEEFEEHEENTTKPYLPSRALTRQQVRALQDGAEELYEAVKNAADPAYLEGYFSEE  
QLRALNNHRQMLNDKKQAQI QLEIRKAMESAEQKEQGLSRDVTWKL RIVSYSKKEKDSVILS  
IWRPSSDLYSLLTEGKRYRIYHLATSKSKSERANI QLAATKKTQYQQLPVSDEILFQIYQPR  
EPLHFSKFLPDFQPSCSEVDLIGFVVSVVKTGLAPFVYLSDECYNLLAIKFWI DLNEDI IKP  
HMLIAASNLQWRPESKSGLLTFLAGDFSVSASPKEGHFQETFNKMKT VENIDILCNEAENKL  
MHILHANDPKWSTPTKDCTSGPYTAQIIPGTGNKLLMSSPNCEIYYQSPLSLCMAKRKS VSTPV  
SAQM TSKSCKGEKEIDDQKNCKRRA LDFLSRLPLPPVSPICTFVSPAAQKAFQPPRSCG TKY  
ETPIKKKELNSPQMTPFKKFNEISLLESNSIADEELALINTQALLSGSTGEKQFISVSESTRTA  
PTSSEDYLR LKRRCTTS LIKEQESSQASTEECEK NKQDTITTKYI

**Chart 1. Amino acid sequences of different fragments of human BRCA2 protein tested for their abilities to bind to the components of the ISGF3 complex.**

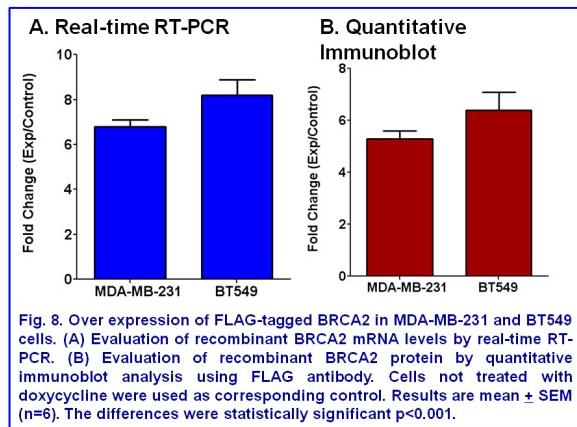
**2. Evaluation of direct binding between BRCA2 and ISGF3 components by yeast 2-hybrid analysis.** We employed BD Clontech Matchmaker Gold yeast two hybrid system for this purpose. We cloned the five BRCA2 ORF fragments described in the previous section into pGBKT7 DNA-BD cloning vector. Similarly we have cloned IRF9 and Stat-2 (wild type and Y690A mutant) ORFs into pGADT7 AD cloning vector. We are also cloned Stat-1 ORFs into pGADT7 vector. The yeast cells were co-transfected individually with pGADT7 fusion construct and pGBKT7 fusion construct. Yeast cells were plated onto synthetic dropout medium lacking leucine, tryptophan, and histidine in the presence of 5-bromo-4-chloro-3-indolyl-ft-D-galactopyranoside (X- $\alpha$ -Gal; Clontech) to select for yeast containing weaker interacting proteins. Yeast cells were also plated onto synthetic dropout medium lacking leucine, tryptophan, histidine, and adenine in the presence of 5-bromo-4-chloro-3-indolyl-ft-D-galactopyranoside (X- $\alpha$ -Gal; Clontech) to select for yeast containing stronger interacting proteins. The positive control (supplied with the reagent kit) used was SV40 T-antigen and p53, known to interact very strongly. The negative controls used were cells co-transfected with empty pGADT7/pGBKT7 vector, untransformed AH109 cells and singly transformed yeast cells.  $\beta$ -Galactosidase assays were done using the Beta Glo Assay reagents and protocols (Promega) to detect  $\beta$ -Galactosidase activity in the co-transfected yeast cell extracts. Yeast cells as well as the Beta-Glo reagent were brought to room temperature. Equal volumes of the reagent and the yeast cell culture were added together. The sample contents were mixed for 30 seconds. Samples were then incubated for 30 min at room temperature and the luciferase activity was measured using a luminometer. Both the cell growth data (not shown) as well as the beta-glo assay data (Fig. 7) suggest that none of the five BRCA2 fragments directly binds to the individual component proteins of the ISGF3 complex in the yeast cells. These data may suggest that BRCA2 perhaps interacts with the ISGF3 complex once it is formed as a trimer.



component proteins of the ISGF3 complex in the yeast cells. These data may suggest that BRCA2 perhaps interacts with the ISGF3 complex once it is formed as a trimer.

**3. Generation of MDA-MB-231 and BT-549 cell derivatives inducibly over expressing BRCA2 and their characterization.** We have generated lentiviral constructs for full length BRCA2 with a C-terminal FLAG tag, as described before for SLUG [20, 21]. We transfected MDA-MB-231 and BT549 cells constitutively expressing tet-repressor protein with this construct, selected for stable transfectants and then evaluated the mRNA and protein levels of recombinant BRCA2 in the presence or absence of doxycycline. We evaluated recombinant BRCA2 mRNA levels with primers designed from BRCA2 ORF and the FLAG epitope. These primers did not amplify the native BRCA2 in the cells. We performed real-time RT-PCR analysis for this evaluation. Total RNA was isolated from the cultured cells using TRIzol reagent (Invitrogen). The cDNA was synthesized from 1  $\mu$ g of total RNA using the iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR quantification was performed following standard protocols using Syber green dye (BioRad). RT-PCR was performed in the iCycler (BioRad): 95°C for 10 min, 40 cycles of 15 s at 95°C, 30 s at 51°C, 30 s at 72°C followed by 1 min at 95°C, 30 s at 55°C and 30 s at 95°C. The fold change over control samples was calculated using  $CT$ ,  $\Delta CT$ , and  $\Delta\Delta CT$  values [20, 21].  $\beta$ -Actin RNA was used as an endogenous control. For the

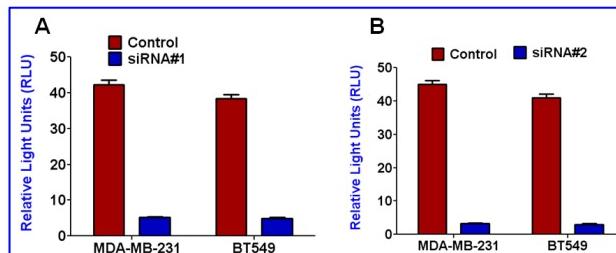
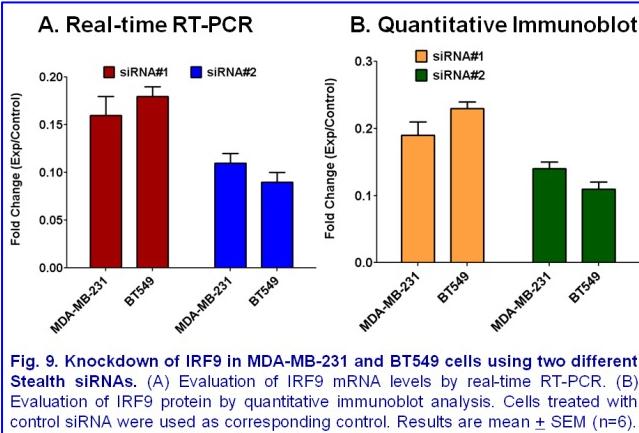
evaluation of BRCA2 protein levels in the breast cancer cell lines by Western blotting with FLAG antibody, protein bands were developed using IR Dye 800 conjugated secondary antibody (LI-COR Biosciences), and visualized using LI-COR's Odyssey Infrared Imaging System. Quantitation and analysis of bands were performed using Odyssey's software.  $\beta$ -actin was used as normalization control. Figs. 8A and 8B show the over expression of recombinant BRCA2 mRNA and protein in these stably transfected cells.



software (Invitrogen) and purchased from Invitrogen and respective control RNAs used in GAGCUCUUCAGAACCGCCUACUUCU-3'/5'-AGAAGUAGGC GGUUCUGAAGA GCUC-3'; Control961: 5'-GAGUCCUG AAACCCGUCCAUCUCU-3'/5'-AGAG AAUGGACGGGUUCAGGACUC-3'; Stealth1025: 5'-CACCGAAGUUC CAGGU AACACUGAA-3'/5'-UUCAGUGUUACC UGGAACUUCGGUG-3'; Control1025: 5'-CACGAACUUGACAUGACAUC CGGAA-3'/5'-UUCCGGAUGUCAUGUCAAGUU CGUG-3'. Transfection of these siRNAs into the breast cells was done by lipofection using the Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Briefly,

#### 4. Knockdown of IRF9 gene expression in the control and the BRCA2-over expressing cells.

IRF9 siRNAs and corresponding control siRNAs were designed using the Block-IT RNAi designer (Invitrogen). The nucleotide sequences of these siRNAs in this study are as follows: Stealth961: 5'-

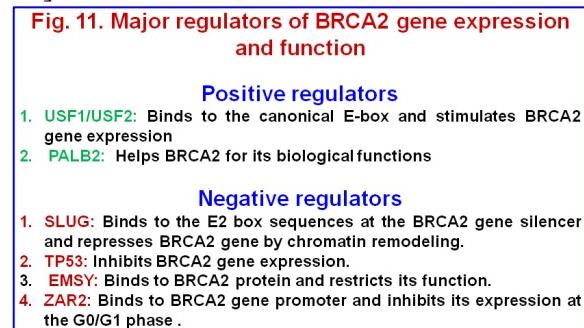


**Fig. 10.** Evaluation of the effect of IRF9 knockdown in two different human breast cancer cell lines on the ISG15 promoter activity. ISG15 gene expression is dependent upon the functional IRF9. Thus knockdown of IRF9 should decrease the activity of ISG15 gene promoter. (A) Data with Stealth RNA#1; (B) Data with Stealth RNA#2. Controls indicate corresponding control siRNAs. Results are mean  $\pm$  SEM ( $n=6$ ). The differences were statistically significant  $p<0.001$ .

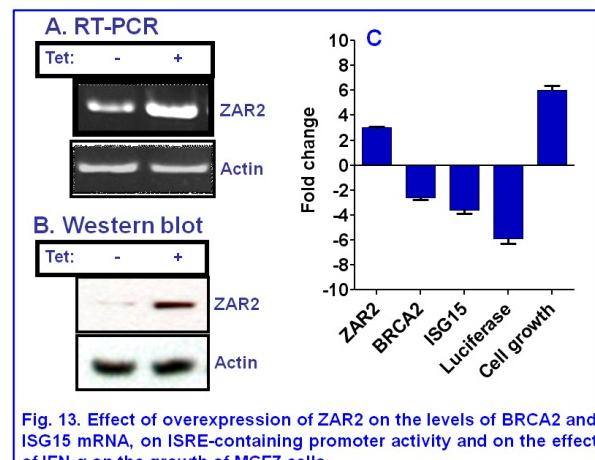
MDA-MB-231 and BT549 cells with the Stealth siRNAs used. Fig. 10 shows the inhibition of the ISG15 gene promoter activity in the IRF9 knocked down cells. We evaluated ISG15 gene promoter activity in transiently transfected cells by dual luciferase assay [20, 21]. We PCR

cells were transfected at  $\sim$ 50% confluence using 100 pmol siRNA in six well plates and whole-cell lysates were prepared 48 h after transfection. We isolated RNA from these cells using Trizol reagents (Invitrogen). Knockdown of the expressions of the target mRNAs by the experimental siRNA and the corresponding protein were verified by real-time RT-PCR and immunoblot analysis, respectively [20, 21] as described in the previous section. Our data (Fig. 9A and 9B) shows the successful knockdown of IRF9 mRNA (Fig. 9A) and protein (Fig. 9B) in the siRNAs used.

amplified human ISG15 gene promoter from total DNA isolated from MDA-MB-231 cells with specific primers. The amplified DNA was cloned into the pCR4.0/TOPO vector (Invitrogen) and subsequently subcloned into the Hind III/Pst I sites of pRL-Null vector (Promega). Cells were seeded on 24-well tissue culture plates in triplicate and allowed to grow overnight to reach 90% to 95% confluence. The following day cells were transfected with pGL3-Control and pRL-ISG15 promoter construct using Lipofectamine 2000 transfection reagent (Invitrogen). Forty-eight hours later, luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) [20, 21]. *Renilla* Luciferase activity was normalized to firefly luciferase activity [20, 21].



regulator for BRCA2 we discovered is the C2H2 type transcriptional repressor SLUG [20]. We found that ectopic expression of ZAR2 or SLUG in MCF7 cells knockdown BRCA2 along with ISG15 (Figs. 13 and 14). Interferon- $\alpha$  (1000 IU/ml) induced stimulation of ISRE-containing promoter activity as well as IFN- $\alpha$  induced growth inhibition were also reduced in the



ZAR2 and SLUG expressing cells (Figs. 13 and 14).

**Task#3**  
**Evaluation of the antiproliferative effects of type I interferons against tumors developed by BRCA2 positive and BRCA2 negative cells in the nude mice xenograft model. (Months 25-36)**

**5. Knockdown of BRCA2 levels in the MCF7 cells by overexpressing ZAR2 or SLUG, made these cells resistant to IFN- $\alpha$ .** BRCA2 is known to be regulated by several factors (Fig. 11). One of the major regulators we recently discovered is ZAR2 [22]. This C4-type zinc finger protein is expressed from the reverse promoter activity of BRCA2 gene (Fig. 12) and negatively reregulates BRCA2 gene expression [22]. Another negative

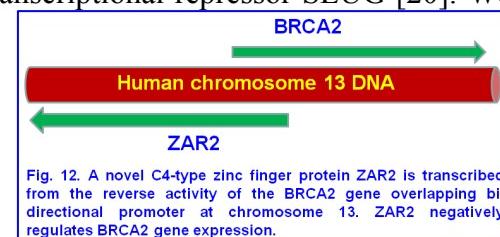
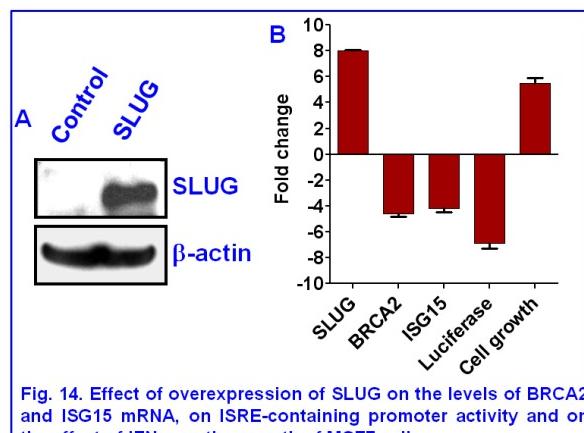


Fig. 12. A novel C4-type zinc finger protein ZAR2 is transcribed from the reverse activity of the BRCA2 gene overlapping bi-directional promoter at chromosome 13. ZAR2 negatively regulates BRCA2 gene expression.

stimulation of ISRE-containing promoter activity as well as IFN- $\alpha$  induced growth inhibition were also reduced in the



## 1. Development of MDA-MB-231 cells expressing shRNA against BRCA2 and their characterization. (Months 25-27).

For inducible knockdown of BRCA2 in MCF7, MDA-MB-231 and BT549 cells, we employed pLKO-Tet-On plasmid (Fig. 15; Addgene). This single plasmid has all the necessary components for the inducible expression of shRNA in target cells. In the absence of doxycycline, shRNA expression is repressed by the TetR protein constitutively expressed also from this plasmid. The cells were treated with 1000 IU/ml of IFN- $\alpha$  for 24 h. Six independent BRCA2-knocked down cell populations and corresponding control shRNA construct-transfected cells were used. We evaluated the levels of mRNAs and proteins of several IFN-regulated genes in these cells.

RNA levels were assayed by real-time RT-PCR. Beta-actin was used as normalization control. GAPDH and BRCA1 were used as references. For protein evaluation Bands were developed using IR Dye-conjugated secondary antibody (LI-COR Biosciences), and visualized using LI-COR's Odyssey Infrared Imaging System. Quantitation and analysis of bands were performed using Odyssey's software. The levels of the mRNAs

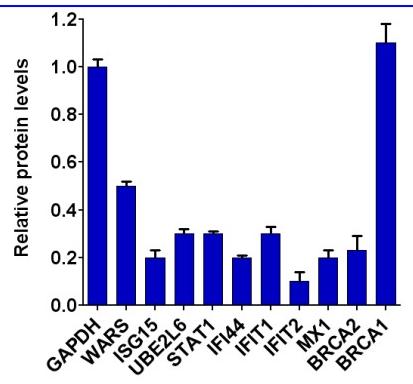


Fig. 17. Evaluation of several IFN regulated protein levels in MDA-MB-231 cells with or without knockdown of BRCA2. Results are mean  $\pm$  SEM ( $n=6$ ). Fold changes observed were statistically significant ( $p<0.001$ ).

and the proteins of WARS, ISG15, UBE2L6, STAT1, IFI44, IFIT1, IFIT2 and MX1 were decreased significantly upon knockdown of BRCA2 in the cells (Figs. 16 and 17). There was no change in the levels of these mRNAs and proteins in the absence of doxycycline. In the presence of doxycycline and IFN- $\alpha$ , the activities of ISG15 and UBE2L6 gene promoters were also inhibited in the BRCA2-knocked down cells (Figs. 18 and 19).

## 2. Establishment of subcutaneous tumors in nude mice with wild type, or BRCA2 knocked down MDA-MB-231 cells. (Months 28-31).

We stably transfected MDA-MB-231 and BT549 cells with pCMV-tdTomato plasmid (Clontech) to express the red tdTomato protein in these cells (Fig. 20). We developed the tumor in BALB/c nude mice with human breast cancer cell MDA-MB-231 with or without knockdown of BRCA2 in these cells.  $5 \times 10^6$  MDA-MB-231 cells were injected into the mammary fat pad of nude mice as described [23]. Tumor developed after 6 weeks.

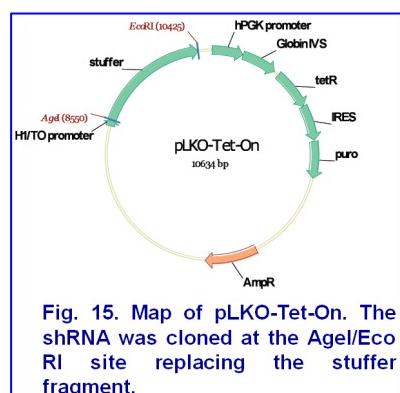


Fig. 15. Map of pLKO-Tet-On. The shRNA was cloned at the Agel/Eco RI site replacing the stuffer fragment.

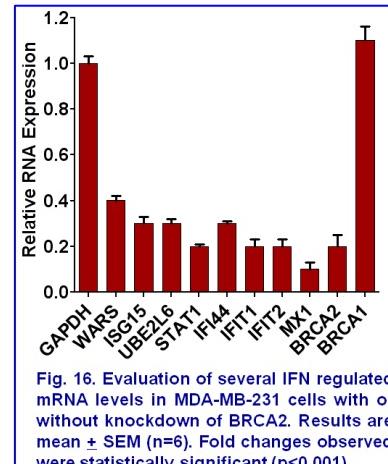


Fig. 16. Evaluation of several IFN regulated mRNA levels in MDA-MB-231 cells with or without knockdown of BRCA2. Results are mean  $\pm$  SEM ( $n=6$ ). Fold changes observed were statistically significant ( $p<0.001$ ).

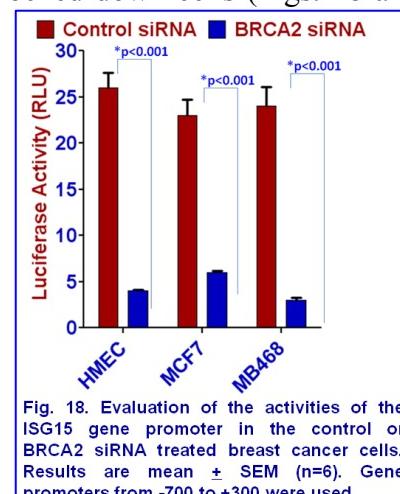


Fig. 18. Evaluation of the activities of the ISG15 gene promoter in the control or BRCA2 siRNA treated breast cancer cells. Results are mean  $\pm$  SEM ( $n=6$ ). Gene promoters from -700 to +300 were used.

**3. Interferon treatment of the mice. (Months 32-34).** We tested the usefulness of the transferrin-coated liposomes in delivering interferon-alpha to the experimental tumors *in vivo* (Fig. 21). This techniques involves the use of transferrin (TF)-pendant-type polyethyleneglycol (PEG)-liposomes (TF-PEG-liposomes), in which TF was covalently linked to the distal terminal of PEG chains on the external surface of PEG-liposomes as a carrier for *in vivo* cytoplasmic targeting to the tumor cells [24, 25] (Fig. 21). TF is a glycoprotein, which transports ferric ion in the body. It is known that the TF receptor concentration on tumor cells is much higher than that on normal cells [24, 25]. The tumor bearing mice were divided into three groups of 5 animals each. One group was control

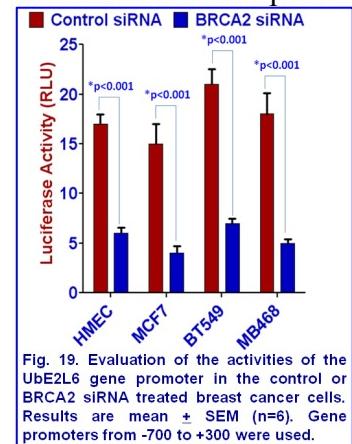


Fig. 19. Evaluation of the activities of the UBE2L6 gene promoter in the control or BRCA2 siRNA treated breast cancer cells. Results are mean  $\pm$  SEM ( $n=6$ ). Gene promoters from -700 to +300 were used.

animals that did not receive any treatment. The next two groups received transferrin-coated liposomes with or without IFN- $\alpha$  (200 IU per gm animal body weight) intravenously through the tail vein [23]. For one set of experiments, tumors were excised from the animals after 1 week post-treatment and the tumor cells were analyzed for ISG15, STAT1, IFI44, IFIT1,

IFI44L, MX1 mRNA and protein levels. Our results indicated that the transferrin-coated liposomes containing IFN- $\alpha$  were efficient in stimulating the interferon-stimulated genes tested in the tumor cells transfected with control pLKO-Tet-On but not with the BRCA2 shRNA containing plasmid *in vivo* (Figs. 22 and 23).

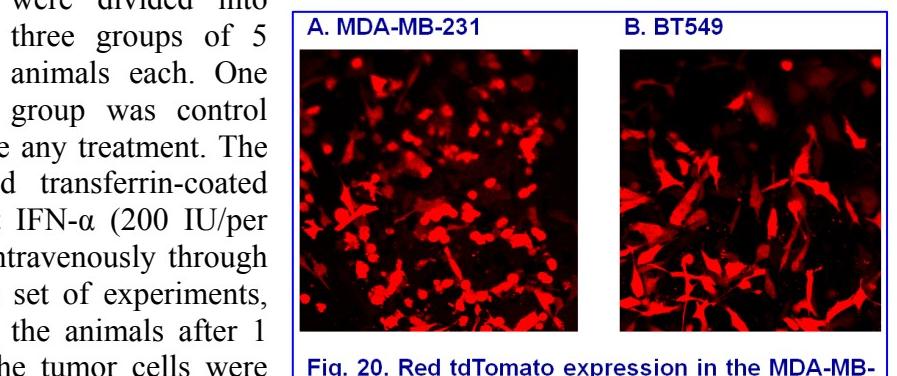


Fig. 20. Red tdTomato expression in the MDA-MB-231 and BT549 cells.

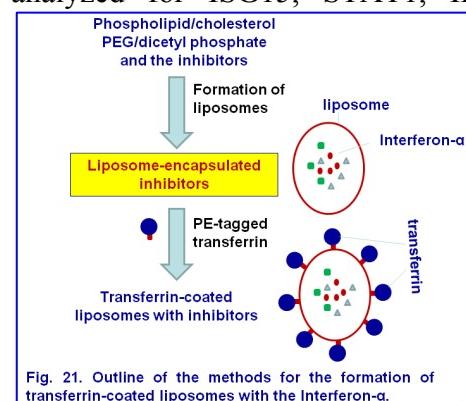


Fig. 21. Outline of the methods for the formation of transferrin-coated liposomes with the Interferon- $\alpha$ .

were performed 6 times (for 3 weeks). Tumor xenografts were measured with calipers twice a week, and tumor volumes were determined using the formula:  $(\text{length} \times \text{width}^2) \times (\pi/6)$ . Fifty-60 days after the last treatment, the animals will be anesthetized with 3% isoflurane-air mixture and killed by cervical dislocation. Tumor samples were stained with hematoxylin and eosin for morphologic observation. The data are being averaged from at least 2 independent experiments. A paired two-tailed *t*-test were used for analysis of the data. Values of  $P < 0.05$  are considered significant. The expression of tdTomato in the tumor forming cells (see above) allowed us to locate tumor cells and to measure tumor growth by utilizing Carestrom *in vivo* imaging system at Meharry small animal

4. Analysis of the excised tumors for weights and histochemical parameters. (Months 34-36). This part of the specific aim is ongoing. Treatments

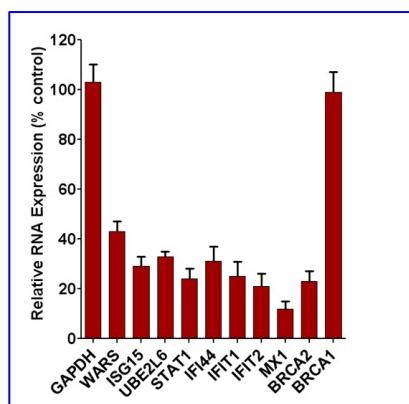
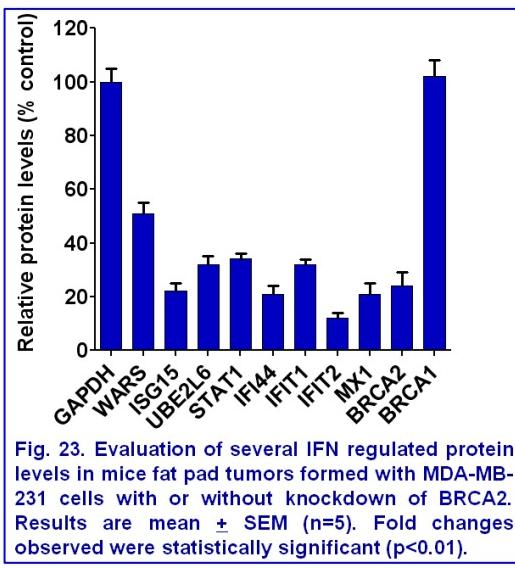


Fig. 22. Evaluation of several IFN regulated mRNA levels in mice fat pad tumors formed with MDA-MB-231 cells with or without knockdown of BRCA2. Results are mean  $\pm$  SEM ( $n=5$ ). Fold changes observed were statistically significant ( $p<0.01$ ).

imaging core facility. Preliminary data indicate significant decrease in the growth of the tumors with BRCA2 knocked down cells.



### Key Research Accomplishments

- N-terminal sequences of human BRCA2 protein appears to be involved in the direct binding of this protein with the ISGF3 protein complex as was revealed by pull down analysis.
- None of the fragments of BRCA2 protein could directly bind with individual components of the ISGF3 complex in the yeast 2-hybrid analysis, suggesting that BRCA2 perhaps binds with the ternary complex after it is formed.
- We were successful in over expressing

FLAG-tagged BRCA2 protein in an inducible manner in MDA-MB-231 and BT549 cells.

- We successfully knocked down IRF9 in the breast cancer cells.
- Knock down of IRF9 negatively affected ISG15 gene promoter activity in the knocked down cells.
- **We derived that ISGF3 ternary complex (P-STAT1/P-STAT2/IRF9) is formed on BRCA2 and the quaternary complex is imported to the nucleus (Fig. 24).**
- **We also predict that BRCA2 helps loading the ISGF3 complex to the target gene promoter (Fig. 24).**

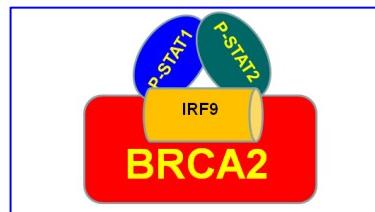


Fig. 24. We derived that ISGF3 ternary complex (P-STAT1/P-STAT2/IRF9) is formed on BRCA2 and the quaternary complex is imported to the nucleus. We also predict that BRCA2 helps loading the ISGF3 complex to the target gene promoter.

### REPORTABLE OUTCOMES:

#### Publication:

1. Mittal M. K., Singh, K., Misra, S. and Chaudhuri, G. (2011) SLUG-induced elevation of D1 cyclin in breast cancer through the inhibition of its ubiquitination. *J. Biol. Chem.* **286**, 469-479. Journal Impact factor: 5.325.
2. Hall, M. 3rd, Misra, S., Chaudhuri, M., and Chaudhuri, G. (2011) Peptide aptamer mimicking RAD51-binding domain of BRCA2 inhibits DNA damage repair and survival in *Trypanosoma brucei*. *Microb. Pathog.* **50**, 252-262. Journal Impact factor: 2.324.
3. Farrow, A. L., Rana, T., Mittal, M. K., Misra, S., and Chaudhuri, G. (2011) *Leishmania*-induced repression of selected non-coding RNA genes containing B-

box element at their promoters in alternatively polarized M2 macrophages. *Mol. Cell. Biochem.* **350**, 47-57. **Journal Impact factor: 1.896.**

4. Rana, T., Misra, S., Mittal, M. K., Farrow, A. L., Wilson, K. T., Linton, M. F., Fazio, S., Willis, I. M., and **Chaudhuri, G.** (2011) Mechanism of down-regulation of RNA polymerase III-transcribed non-coding RNA genes in macrophages by *Leishmania*. *J. Biol. Chem.* **286**, 6614-6626. **Journal Impact factor: 5.325.**
5. Misra, S. Sharma, S., Agarwal, A., Khedkar, S. V., Tripathi, M. K., Mittal M. K., and **Chaudhuri, G.** (2010) Cell cycle-dependent regulation of the bi-directional overlapping promoter of human BRCA2/ZAR2 genes in breast cancer cells. *Mol. Cancer.* **9**, 50. **Journal Impact factor: 4.16.**
6. Ochieng, J., and **Chaudhuri, G.** (2010) Cystatin superfamily. *J. Health Care Poor Underserved.* **21**, 51-70.
7. Mittal, M. K., Myers, J. N., Bailey, C. K., Misra, S., and **Chaudhuri, G.** (2010) Mode of action of the retrogene product SNAI1P, a SNAIL homolog, in human breast cancer cells. *Mol. Biol. Rep.* **37**, 1221-1227. **Journal Impact factor: 2.038.**
8. Kaneko, H., Dridi, S., Tarallo, V., Gelfand, B. D., Fowler, B. J., Cho, W. G., Kleinman, M. E., Ponicsan, S. L., Hauswirth, W. W., Chiodo, V. A., Karikó, K., Yoo, J. W., Lee, D. K., Hadziahmetovic, M., Song, Y., Misra, S., **Chaudhuri, G.**, Buaas, F. W., Braun, R. E., Hinton, D. R., Zhang, Q., Grossniklaus, H. E., Provis, J. M., Madigan, M. C., Milam, A. H., Justice, N. L., Albuquerque, R. J., Blandford, A. D., Bogdanovich, S., Hirano, Y., Witta, J., Fuchs, E., Littman, D. R., Ambati, B. K., Rudin, C. M., Chong, M. M., Provost, P., Kugel, J. F., Goodrich, J. A., Dunaief, J. L., Baffi, J. Z., and Ambati, J. (2011) DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. *Nature.* **471**, 325-330. **Journal Impact factor: 34.48.**

#### Meeting abstracts:

1. Mittal, M. K., Singh, K. and **Chaudhuri, G.** (2009) Mechanisms of SLUG-induced drug resistance development in breast cancer cells. **Presented as poster at the 2009 San Antonio Breast Cancer Symposium (SABCS) held in Henry B. Gonzalez Convention Center, San Antonio, Texas, USA, December 9-13, 2009.**
2. Bailey, C. K., Mittal, Mukul, Misra, Smita and **Chaudhuri, G.** (2010) Reduction of the invasive phenotype of SNAI-over expressing human breast cancer cells by peptide aptamer-mediated inhibition of SNAI protein functions. **Presented at the AACR Annual meeting in Washington DC on April 17-21, 2010.**
3. Mittal, M. K., and **Chaudhuri, G.** (2010) Repression of alpha-, beta- and gamma-catenin gene expressions by SNAI2 in human breast cancer cells. **Presented at the AACR Annual meeting in Washington DC on April 17-21, 2010.**

4. Hall, Mack III, Misra, Smita, and **Chaudhuri, G.** (2010) Molecular analysis of the physical interactions of *Trypanosoma brucei* BRCA2 with different RAD51 isoforms. **Presented at the ASBMB Annual meeting in Anaheim, CA on April 24-28, 2010.**
5. Misra, Smita, and **Chaudhuri, G.** (2010) Regulation of BRCA2 gene expression through CpG methylation of its bi-directional promoter induced by endogenous siRNAs. **Presented at the ASBMB Annual meeting in Anaheim, CA on April 24-28, 2010.**
6. Yarlagadda, V., Misra, S., Mittal, M. K., and **Chaudhuri, G.** (2011) Differential Translational Regulation of SLUG mRNA by a uORF in SLUG-High and SLUG-Low Cancer Cells. **Presented at the ASBMB Annual meeting in Washington DC on April 9-13, 2011.**
7. Misra, S., Mittal, M. K., and **Chaudhuri, G.** (2011) Selective modulation of the redox state and thus the repressor function of SLUG by peroxiredoxin 5 at BRCA2 gene silencer in breast cancer cells. **Presented at the ASBMB Annual meeting in Washington DC on April 9-13, 2011.**
8. Mittal, M. K., Misra, S., and **Chaudhuri, G.** (2011) SLUG-induced elevation of D1 cyclin in breast cancer through the inhibition of its ubiquitination. **Accepted for presentation at the Era of Hope Meeting at Orlando World Center Marriott, Orlando, Florida on Aug 2-5, 2011.**
9. Misra, S., Mittal, M. K., and **Chaudhuri, G.** (2011) Cell cycle-dependent regulation of the bi-directional overlapping promoter of human BRCA2/ZAR2 genes in breast cancer cell. **Accepted for presentation at the Era of Hope Meeting at Orlando World Center Marriott, Orlando, Florida on Aug 2-5, 2011.**
10. **Chaudhuri, G.** Misra, S., and Mittal, M. K. (2011) BRCA2 as a determinant of the efficacy of type I interferons against breast cancer cell proliferation. **Accepted for presentation at the Era of Hope Meeting at Orlando World Center Marriott, Orlando, Florida on Aug 2-5, 2011.**
11. Misra, S., Mittal, M. K., and **Chaudhuri, G.** (2011) Repression of retrograde transport of growth promoting receptors by SLUG in breast tumor cells. **Accepted for presentation at the Era of Hope Meeting at Orlando World Center Marriott, Orlando, Florida on Aug 2-5, 2011.**

### **Conclusion:**

We concluded that ISGF3 ternary complex (P-STAT1/P-STAT2/IRF9) is formed on BRCA2 and the quaternary complex is imported to the nucleus. We also predict that BRCA2 helps loading the ISGF3 complex to the target gene promoter.

### **REFERENCES:**

- [1] Pestka, S., Langer, J. A., Zoon, K. C., and Samuel, C. E. (1987) Interferons and their actions. *Annu. Rev. Biochem.* **56**, 727-777.
- [2] Vilcek, J. (2006). Fifty years of interferon research: aiming at a moving target. *Immunity* **25**, 343–348.
- [3] Pestka, S., Krause, C. D., and Walter, M. R. (2004). Interferons, interferonlike cytokines, and their receptors. *Immunol. Rev.* **202**, 8–32.
- [4] van Boxel-Dezaire, A. H. H., Rani, M. R. S., and Stark, G. R. (2006). Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity* **25**, 361–372.

- [5] Takaoka, A., and Yanai, H. (2006) Interferon signalling network in innate defense. *Cell. Microbiol.* **8**, 907-922.
- [6] Imai, K., and Takaoka, A. (2006) Comparing antibody and small-molecule therapies for cancer. *Nat. Rev. Cancer.* **6**, 714-727.
- [7] Nicolini, A., Carpi, A., and Rossi, G. (2006) Cytokines in breast cancer. *Cytokine Growth Factor Rev.* **17**, 325-337.
- [8] Toma, S., Raffo, P., Nicolo, G., Canavese, G., Margallo, E., Vecchio, C., Dastoli, G., Iacona, I., and Regazzi-Bonora, M. (2000) Biological activity of all-transretinoic acid with and without tamoxifen and alpha-interferon 2a in breast cancer patients. *Int. J. Oncol.* **17**, 991-1000.
- [9] Recchia, F., Frati, L., Rea, S., Torchio, P., and Sica, G. (1998) Minimal residual disease in metastatic breast cancer: treatment with IFN-beta, retinoids, and tamoxifen. *J. Interferon Cytokine Res.* **18**, 41-47.
- [10] Kornek, G., Reiner, A., Sagaster, P., Stierer, M., Mayer, A., and Ludwig, H. (1999) Effect of interferon alpha-2a on hormone receptor status in patients with advanced breast cancer. *Cancer Invest.* **17**, 189-194.
- [11] Tripathi, M. K., and Chaudhuri, G. (2005) Down-regulation of UCRP and UBE2L6 in BRCA2 knocked-down human breast cells. *Biochem. Biophys. Res. Commun.* **328**, 43-48.
- [12] de Veer, M. J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J. M., Silverman, R. H., and Williams, B. R. (2001) Functional classification of interferon-stimulated genes identified using microarrays. *J. Leukoc. Biol.* **69**, 912-920.
- [13] Shamoo, Y. (2003) Structural insights into BRCA2 function. *Curr. Opin. Struct. Biol.* **13**, 206-211.
- [14] Shivji, M. K., and Venkitaraman, A. R. (2004) DNA recombination, chromosomal stability and carcinogenesis: insights into the role of BRCA2. *DNA Repair (Amst).* **3**, 835-843.
- [15] Turner, N., Tutt, A., and Ashworth, A. (2004) Hallmarks of 'BRCAness' in sporadic cancers. *Nat. Rev. Cancer.* **4**, 814-819.
- [16] Rudkin, T. M., and Foulkes, W. D. (2005) BRCA2: breaks, mistakes and failed separations. *Trends Mol. Med.* **11**, 145-148.
- [17] Pellegrini, L., and Venkitaraman, A. (2004) Emerging functions of BRCA2 in DNA recombination. *Trends Biochem. Sci.* **29**, 310-316.
- [18] Rahman, N., and Stratton, M. R. (1998) The genetics of breast cancer susceptibility. *Annu. Rev. Genet.* **32**, 95-121.
- [19] Powell, S. N., Willers, H., and Xia, F. (2002) BRCA2 Keeps Rad51 in line. high-fidelity homologous recombination prevents breast and ovarian cancer? *Mol. Cell* **10**, 1262-1263.
- [20] Tripathi, M. K., Misra, S., Khedkar, S. V., Hamilton, N., Irvin-Wilson, C., Sharan, C., Sealy, L., and Chaudhuri, G. (2005) Regulation of BRCA2 gene expression by the SLUG repressor protein in human breast cells. *J. Biol. Chem.* **280**, 17163-17171.
- [21] Mittal, M., Myers, J. N., Misra, S., Bailey, C. K. and Chaudhuri, G. (2008) *In vivo* binding to and functional repression of the VDR gene promoter by SLUG in human breast cells. *Biochem. Biophys. Res. Commun.* **372**, 30-34.
- [22] Misra, S., Sharma, S., Agarwal, A., Khedkar, S. V., Tripathi, M. K., Mittal M. K., and Chaudhuri, G. (2010) Cell cycle-dependent regulation of the bi-directional overlapping promoter of human BRCA2/ZAR2 genes in breast cancer cells. *Molecular Cancer* **9**, 50.
- [23] Viloria-Petit AM, David L, Jia JY, Erdemir T, Bane AL, Pinnaduwage D, Roncari L, Narimatsu M, Bose R, Moffat J, Wong JW, Kerbel RS, O'Malley FP, Andrulis IL, Wrana

JL. A role for the TGFbeta-Par6 polarity pathway in breast cancer progression. Proc Natl Acad Sci U S A. 2009; 106:14028-33. PubMed Central PMCID: PMC2729014.

- [24] Zheng Y, Yu B, Weecharangsan W, Piao L, Darby M, Mao Y, Koynova R, Yang X, Li H, Xu S, Lee LJ, Sugimoto Y, Brueggemeier RW, Lee RJ. Transferrin-conjugated lipid-coated PLGA nanoparticles for targeted delivery of aromatase inhibitor 7alpha-APTADD to breast cancer cells. Int J Pharm. 2010; 390:234-41. PubMed PMID: 20156537.
- [25] Ishida O, Maruyama K, Tanahashi H, Iwatsuru M, Sasaki K, Eriguchi M, Yanagie H. Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors *in vivo*. Pharm Res. 2001; 18: 1042-8. PubMed PMID: 11496943.

APPENDICES: None.